HUMAN HEMATOPOIETIC GROWTH REGULATORY GENE AND USES

BACKGROUND OF THE INVENTION

1.   Field of the Invention

This invention relates to a human hematopoietic growth regulatory gene termed *hiwi* and genes corresponding thereto. Specifically, the invention relates to the isolation, cloning and sequencing of complementary DNA (cDNA) copies of messenger RNA (mRNA) encoding a novel human *hiwi* gene. The invention also relates to the construction of recombinant expression constructs comprising cDNA of the novel human *hiwi* gene, said recombinant expression constructs being capable of expressing *hiwi* gene product in cultures of transformed prokaryotic and eukaryotic cells. Production of the *hiwi* gene product in such cultures is also provided. The invention relates to the use of such cultures of such transformed cells to produce homogeneous compositions of the human *hiwi* gene product. The invention also provides cultures of such cells producing the *hiwi* protein for the characterization of novel and useful drugs. Antibodies against and epitopes of this novel human *hiwi* gene product are also provided by the invention. Methods for isolating human hematopoietic stem cells from biological samples such as bone marrow are also provided.

2.   Background of the Invention

Stem cells can undergo self-renewal as well as generate differentiated progeny. Hematopoietic stem cells (HSC) have the ability to undergo self-renewal and to differentiate into cells belonging to multiple hematopoietic lineages (Morrison *et al.*, 1995, *Annu Rev Cell Dev Biol.* 11:35-71; Chen *et al.*, 1997, *Immunol Rev.* 157:41-51).

The capacity of a hematopoietic stem cell to remain undifferentiated and be capable of reconstituting a myeloablated host as well as its ability to generate multiple differentiated cell types is central to its pivotal role in normal hematopoiesis. These properties allow stem cells to maintain hematopoiesis throughout the lifespan of an organism.

The knowledge of the behavior of HSCs is limited due to their rarity, difficulty of efficient isolation, and their sensitivity to manipulation (Morrison *et al., ibid.*; Chen *et al., ibid.*). Despite an improved ability of various laboratories to isolate and manipulate pure populations of murine and human HSCs (Goodell, 1999, *Blood* 94:2545-2547; Huang *et al.*, 1999, *Blood* 94:2595-2604) current understanding of mechanisms by which a stem cell divides and retains its unique biological properties has eluded the efforts of a large number of investigators (Vaziri *et al.*, 1994, *Proc Natl Acad Sci. U S A* 91:9857-9860; Lansdorp *et al.*, 1995, *Exp Hematol.* 23:187-191; van der Loo *et al.*, 1995, *Blood* 85:2598-2606; Peters *et al.*, 1995, *Exp Hematol.* 23:461-469; Peters *et al.*, 1996, *Blood* 87:30-37; Yonemura *et al.*, 1996, *Proc Natl Acad Sci. U S A* 93:4040-4044).

Elucidation of the genetic program that underlies the unique biological properties of HSCs has been the focus of a growing number of laboratory groups (Vaziri *et al., ibid.*; Lansdorp *et al., ibid.*) using a variety of approaches. Array technology, for instance, now permits simultaneous monitoring of expression patterns of thousands of genes during cellular differentiation and response (van der Loo *et al., ibid.*; Peters *et al., ibid.*). The key to the successful implementation of such technology to the study of stem cell biology is the development of the means to assign priority to such genes and to determine their function.

The self-renewal capacity of several classes of stem cells is thought to be controlled by external signals and intrinsic cellular processes (Morrison *et al., ibid.*; Chen *et al., ibid.*; Bruno *et al.*, 1995, *Exp Hematol.* 23:1212-1217; Hoffman, 1999, *Curr Opin Hematol.* 6:184-191). Over the last 2 decades, a variety of external stimuli (cytokines, matrix proteins) that alter HSC self-renewal have been the subject of intense investigation. Although a number of such external signals that interact with specific receptors on HSC have been identified, the signaling mechanisms that govern HSC self-renewal have eluded investigation.

A different approach to analyze the genetic organization of human HSCs is to analyze expression of genes originally shown to affect stem cell development in lower species (Peters *et al., ibid.*; Yonemura *et al., ibid.*; Zon 1995, *Blood* 86:2876-2891). In these experiments, genes that were originally shown to affect stem cell development in lower species have been shown subsequently to be expressed by human hematopoietic cells and to have profound regulatory effect on human hematopoiesis. Lower organisms such as *Drosophilae, C. elegans* and *D. rerio* (zebra

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fish) have been utilized as effective models for studying mechanisms that are conserved among diverse developmental systems (Lewis, 1978, *Nature* 276:565-570; Zon, 1995, *ibid.*; Tabara *et al.*, 1999, Cell 99:123-132). Studies from *Xenopus*, for instance, have revealed a multitude of genes involved in mesoderm induction including members of the transforming growth factor β superfamily, fibroblast growth factor and at least 19 members of the Wnt gene family have been identified in diverse species ranging from roundworm and insects to humans (Sidow, 1992, *Proc Natl Acad Sci. USA* 89:5098-5102; Austin *et al.*, 1997, *Blood* 89:3624-3635). Wnt gene family members have subsequently been shown to have profound effects on murine and human hematopoiesis.

Intrinsic cellular mechanisms that regulate stem cell self-renewal have been explored in a variety of model systems including germ line stem cells (GSCs) in several lower species. *Drosophila* has been a particularly useful model for studying biological processes that are conserved in higher developmental systems (Lewis, *ibid.*; Nusslein-Volhard *et al.*, 1980, *Nature* 287:795-801; Lin & Spradling, 1993, *Dev Biol.* 159:140-152; Lin *et al.*, 1997, *Development* 124:2463-2476; Lin, 1998, *Curr Opin Cell Biol.* 10:687-693; Cox *et al.*, 1998, *Genes Dev.* 12:3715-3727; Lin, 1999, *Annu Rev Genet.* 31:455-491; Benfey, 1999, *Curr Biol.* R171). In *Drosophila*, stem cells exist in the germ line at the apical tip of each ovariole, the germarium, which is the functional portion of the ovary (Lin & Spradling, *ibid.*; King, 1970, OVARIAN DEVELOPMENT IN *DROSOPHILA MELANOGASTER*," New York: McGraw-Hill). Each ovary consists of 10-17 ovarioles. Each germarium contains 2 to 3 GSCs that are in direct contact with specialized somatic cells, the basal terminal filament cells (King, 1970, *ibid.*; Lin, 1998, *ibid*; Lin, 1999, *ibid.*). GSCs undergo asymmetric divisions to produce daughter stem cells and a differentiated daughter cell, a cystoblast. The GSCs provide a continuous source of totipotent cells for the production of gametes needed for fertilization (Lin, 1999, *ibid.*). They are very similar to HSCs in their ability to not only self-renew but to remain capable of generating large numbers of differentiated daughter cells (Lin, 1999, *ibid.;* Benfey, 1999, *ibid.*). The intracellular mechanisms which serve as the determinants of asymmetric-segregating cell fates of GSCs depend not only on the basic cell cycle machinery but also on a family of recently identified genes, some of which are evolutionarily conserved (Cox *et al.*, 1998, *ibid.;* Benfey, 1999, *ibid,*). A group of somatic cells in *Drosophila*, termed terminal filament cells, which are distal and immediately adjacent to the GSCs, have

been shown to regulate GSC division (Lin, 1998, *ibid.;* Lin, 1999, *ibid.;* Lin & Spradling, *ibid*). Laser ablation of the terminal filament increases the rate of oogenesis by 40% (Lin & Spradling, *ibid.*).

Recently a number of genes including *dpp, piwi, pumilio* and *fs(1)Yb* have been identified and shown to be essential for GSC maintenance (Lin & Spradling, *ibid.;* Cox *et al.*, 1998, *ibid.;* King & Lin, 1999, D*evelopment* <u>126</u>:1833-1844). Among these genes, *piwi* has been of special interest. It has recently has been demonstrated to be an essential stem cell gene in *Drosophila* and *C. elegans* and to be expressed in tissues belonging to many species including human. The *Drosophila piwi* gene is required for asymmetric division of GSCs but is not required for differentiation of committed daughter cells. *piwi* expression in adjacent somatic cells, terminal filament cells, regulates GSC division (Cox *et al.*, 1998, *ibid.*). Loss-of-function mutations in the *piwi* gene found in the terminal filament leads to a failure of stem cell maintenanc3 (Lin & Spradling, *ibid.;* Cox *et al.*, 1998, *ibid.*); *piwi* is expressed not only in the terminal filament but also in the germ line. Loss of *piwi* function in the germ line, however, is not known to affect GSC division. The protein encoded by *piwi* is extraordinarily well conserved along the evolutionary tree, being found in both *Caenorhabditis elegans* and primates (Cox *et al.*, 1998, *ibid*).

Thus, there is a need in the art to identify genes and gene products in hematopoietic stem cells that regulate cell cycling and proliferation. There further is a need in the art to identify a human homolog for the *Drosophila piwi* gene to determine the role of said homolog in hematopoietic stem cell development and maintenance. There is also a need in the art to develop drugs and other active agents for controlling, promoting or inhibiting hematopoietic stem cell growth, proliferation and differentiation to permit manipulation of hematopoietic stem cells and provide renewable sources of said stem cells. There is additionally a need for developing compounds to inhibit leukemia cell growth and induce apoptosis of such cells as a means of cancer treatment.

SUMMARY OF THE INVENTION

The present invention relates to the cloning, expression and functional characterization of a human hematopoietic growth regulatory gene termed *hiwi*. The invention comprises nucleic acids having a nucleotide sequence of a novel human *hiwi*

gene. The nucleic acids provided by the invention comprise a complementary DNA (cDNA) copy of the corresponding mRNA transcribed *in vivo* from the human *hiwi* gene of the invention. In one preferred embodiment, the human *hiwi* gene encodes a protein having an amino acid sequence identified by SEQ ID NO. 2. In another

5   preferred embodiment, the human *hiwi* gene has a nucleic acid sequence identified by SEQ ID NO. 1. Also provided are the deduced amino acid sequence of the cognate proteins of the cDNAs provided by the invention, methods of making said cognate proteins by expressing the cDNAs in cells transformed with recombinant expression constructs comprising said cDNAs, and said recombinant expression constructs and

10   cells transformed thereby.

This invention in a first aspect provides nucleic acids, nucleic acid hybridization probes, recombinant eukaryotic expression constructs capable of expressing the human *hiwi* gene of the invention in cultures of transformed cells, and such cultures of transformed eukaryotic cells that synthesize the human *hiwi* gene. In

15   another aspect, the invention provides homogeneous compositions of the human *hiwi* gene product of the invention, and membrane and cytosolic preparations from cells expressing the human *hiwi* gene product, as well as antibodies against and epitopes of the human *hiwi* gene product. The invention in another aspect provides methods for making said homogenous preparations and membrane and cytosolic preparations

20   using cells transformed with the recombinant expression constructs of the invention and expressing said human *hiwi* gene product thereby. Methods for characterizing the biochemical properties of the human *hiwi* gene product and methods for using these proteins in the development of agents having pharmacological uses related to the *hiwi* gene product are also provided by the invention.

25   In a first aspect, the invention provides a nucleic acid having a nucleotide sequence encoding a human *hiwi* gene. In a preferred embodiment, the nucleic acid encodes a nucleotide sequence comprising 2328 basepairs (bp) encoding a776 amino acid sequence. In this embodiment of the invention, the nucleotide sequence of the human *hiwi* gene is the nucleotide sequence depicted in Figure 1 (SEQ ID No: 1).

30   The sequence shown in Figure 1 will be understood to represent one specific embodiment of a multiplicity of nucleotide sequences that encode the human *hiwi* gene amino acid sequence (SEQ ID No.: 2) of the invention and that these different nucleotide sequences are functionally equivalent and are intended to be encompassed by the claimed invention. In addition, it will be understood that different organisms

and cells derived therefrom express preferentially certain tRNAs corresponding to subsets of the degenerate collection of tRNAs capable of encoding certain of the naturally-occurring amino acids, and that embodiments of the multiplicity of nucleotide sequences encoding the amino acid sequence of the human *hiwi* gene product of the invention that are optimized for expression in specific prokaryotic and eukaryotic cells are also encompassed by the claimed invention. Isolated nucleic acid derived from human genomic DNA and isolated by conventional methods using the human cDNA provided by the invention is also within the scope of the claimed invention. Finally, it will be understood that allelic variations of the human *hiwi* gene, including naturally occurring and *in vitro* modifications thereof are within the scope of this invention. Each such variant will be understood to have essentially the same amino acid sequence as the sequence of the human *hiwi* gene product disclosed herein.

The human *hiwi* gene product corresponding to the human cDNA of the invention is a second aspect of the claimed invention. In a preferred embodiment, the human *hiwi* gene is encoded by a nucleic acid having a deduced amino acid sequence shown in Figure 1 (SEQ ID No.: 2). Also provided are preparations of said human *hiwi* gene product comprising a membrane or cytosolic preparation from a cell, most preferably a recombinant cell, expressing a nucleic acid encoding a human *hiwi* gene.

As provided in this aspect of the invention is a homogeneous composition of a human *hiwi* gene product having a molecular weight of about 90kD or derivative thereof and having an amino acid sequence shown in Figure 1 and identified by SEQ ID No.: 2, said size being understood to be the predicted size of the protein before any post-translational modifications thereof.

This invention provides both nucleotide and amino acid probes derived from the sequences herein provided. The invention includes probes isolated from either cDNA or genomic DNA, as well as probes made synthetically with the sequence information derived therefrom. The invention specifically includes but is not limited to oligonucleotide, nick-translated, random primed, or *in vitro* amplified probes made using cDNA or genomic clone of the invention encoding a human *hiwi* gene or fragment thereof, and oligonucleotide and other synthetic probes synthesized chemically using the nucleotide sequence information of cDNA or genomic clone embodiments of the invention.

It is a further object of this invention to provide such nucleic acid hybridization probes to determine the pattern, amount and extent of expression of the

human *hiwi* gene in various tissues of mammals, including humans. It is also an object of the present invention to provide nucleic acid hybridization probes derived from the sequences of human *hiwi* gene of the invention to be used for the detection and diagnosis of diseases and pathological conditions associated with differential

5   expression of the human *hiwi* gene. It is an object of this invention to provide nucleic acid hybridization probes derived from the nucleic acid sequences of the human *hiwi* gene herein disclosed to be used for the detection of novel related genes.

The present invention also includes synthetic peptides made using the nucleotide sequence information comprising the cDNA embodiments of the invention.

10   The invention includes either naturally occurring or synthetic peptides which may be used as antigens for the production of human *hiwi* gene product-specific antibodies, or useful as competitors of human *hiwi* gene product molecules for agonist, antagonist or drug binding, or to be used for the production of inhibitors of binding with agonists, antagonists, analogues thereof or any other binding partner.

15   The present invention also provides antibodies against and epitopes of the human *hiwi* gene product molecules of the invention. It is an object of the present invention to provide antibodies that are immunologically reactive to the human *hiwi* gene product. It is a particular object to provide monoclonal antibodies against the human *hiwi* gene product. Hybridoma cell lines producing such antibodies are also

20   objects of the invention. It is envisioned that such hybridoma cell lines may be produced as the result of fusion between a non-immunoglobulin producing mouse myeloma cell line and spleen cells derived from a mouse immunized with a cell line which expresses antigens or epitopes of a human *hiwi* gene product of the invention. The present invention also provides hybridoma cell lines that produce such antibodies,

25   and can be injected into a living mouse to provide an ascites fluid from the mouse that is comprised of such antibodies. It is a further object of the invention to provide immunologically-active epitopes of the human *hiwi* gene product. Chimeric antibodies immunologically reactive against the human *hiwi* gene product are also within the scope of this invention.

30   The present invention provides recombinant expression constructs comprising a nucleic acid encoding a human *hiwi* gene of the invention wherein the construct is capable of expressing the encoded human *hiwi* gene product in cultures of cells transformed with the construct. A preferred embodiment of such constructs comprises a human *hiwi* gene cDNA depicted in Figure 1 (SEQ ID No.: 1), such constructs being

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capable of expressing the human *hiwi* gene product encoded therein in cells transformed with the construct.

The invention also provides prokaryotic and more preferably eukaryotic cells transformed with the recombinant expression constructs of the invention, each such cells being capable of and indeed expressing the human *hiwi* gene product encoded in the transforming construct, as well as methods for preparing human *hiwi* gene-encoded protein using said transformed cells.

The present invention also includes within its scope protein preparations of prokaryotic and eukaryotic cell membranes comprising the human *hiwi* gene-encoded protein of the invention, derived from cultures of prokaryotic or eukaryotic cells, respectively, transformed with the recombinant expression constructs of the invention.

The present invention also includes within its scope protein preparations of prokaryotic and eukaryotic cytoplasmic fractions containing the human *hiwi* gene – encoded protein of the invention, derived from cultures of prokaryotic or eukaryotic cells, respectively, transformed with the recombinant expression constructs of the invention.

The invention also provides methods identifying a compound that induces or increases *hiwi* gene expression in mammalian cells, preferably a human cell, most preferably a leukemia cell or a hematopoietic stem cell. In these embodiments, the method comprises the steps of culturing a mammalian cell under conditions wherein the cell does not express the *hiwi* gene or expresses an amount of the *hiwi* gene product insufficient to repress cell proliferation; contacting the cell with a test compound for a time period; assaying the cells at intervals during the time period for *hiwi* gene expression and cell proliferation or apoptosis; and identifying compounds that induce *hiwi* gene expression, and concomitantly decrease cell proliferation or increase the percentage of cells undergoing apoptosis or both. Compounds that induce or increase *hiwi* gene expression in mammalian cells, preferably a human cell, most preferably a leukemia cell or a hematopoietic stem cell are also provided by the invention.

The invention also provides methods for maintaining or increasing the percentage of hematopoietic stem cells, preferably human hematopoietic stem cells, and most preferably CD34$^+$ human hematopoietic stem cells, from biological samples comprising said stem cells, most preferably bone marrow samples or peripheral blood samples. The method comprises the step of increasing *hiwi* gene product expression

in said cells. In preferred embodiments, the method comprises the step of culturing the cells in the presence of a *hiwi* gene- inducing compound identified by the methods of the invention. In alternative preferred embodiment, the method comprises the step of introducing into said cells a recombinant expression construct of the invention encoding a *hiwi* gene, most preferably a human *hiwi* gene. In preferred embodiments, the *hiwi* gene encodes a gene product having an amino acid sequence identified by SEQ ID NO. 2. In preferred embodiments, expression of the *hiwi* gene is inducible gene expression.

Specific preferred embodiments of the present invention will become evident from the following more detailed description of certain preferred embodiments and the claims.

DESCRIPTION OF THE DRAWINGS

Figure 1 illustrates the nucleotide (SEQ ID No.: 1) and amino acid (SEQ ID No.: 2) sequences of a human *hiwi* gene product and *Drosophila* piwi protein.

Figure 2A is a fluorescence-activated cell sorting (FACS) analysis of CD34 and CD38 (hi, lo and negative) expressing human adult bone marrow cells.

Figure 2B is a photograph of an ethidium bromide stained gel electrophoretic analysis of DNA fragments produced by reverse transcriptase-polymerase chain reaction (RT-PCR) analysis of RNA isolated from CD34 and CD38 expressing human adult bone marrow cells. Lane 1 is a 123 bp size marker; lane 2 is the fragment produced from $CD34^+CD38^-$ cells; lane 3 is the fragment produced from $CD34^+CD38^-$ cells without reverse transcription of cellular RNA; lane 4 is the fragment produced from $CD34^+CD38^{lo}$ cells; lane 5 is the fragment produced from $CD34^+CD38^{lo}$ cells without reverse transcription of cellular RNA; lane 6 is the fragment produced from $CD34^+CD38^{hi}$ cells; lane 7 is the fragment produced from $CD34^+CD38^{hi}$ cells without reverse transcription of cellular RNA; lane 8 is a negative control (water); lane 9 is a positive control (human testis).

Figures 3A through 3C is a photograph of an ethidium bromide stained gel electrophoretic analysis of DNA fragments produced by RT-PCR analysis of RNA isolated from human leukemia cell lines. In Figure 3A, lane 1 is a 1 kilobase size marker; lane 2 is the fragment produced from TF1 cells; lane 3 is the fragment produced from Jurkat cells; lane 4 is the fragment produced from KG1a cells; lane 5 is

the fragment produced from KG1 cells; lane 6 is the fragment produced from K562 cells; lane 7 is the fragment produced from CEM cells; lane 8 is the fragment produced from BV173 cells; lane 9 is the fragment produced from SUPB13cells; lane 10 is a negative control (water); lane 11 is a positive control (human testis). Figure 3B is PCR analysis of the samples in lanes 2-6 of Figure 3A performed without reverse transcription of cellular RNA. Figure 3C is RT-PCR analysis of a GAPD internal control.

Figure 4 is a graph of MTT exclusion analysis of control KG1 cells, KG1 cells transfected with pCIneo vector and KG1 cells transfected with pCIneo-*hiwi*.

Figures 5A through 5C is a photograph of an ethidium bromide stained gel electrophoretic analysis of DNA fragments produced by RT-PCR analysis of RNA isolated from human leukemia cell lines. In Figure 5A, lane 1 is a 1 kilobase size marker; lanes 2-8 are mock-transfected KG1 cells from days 1-6 and day 9, respectively; lanes 9-15 are vector-transfected KG1 cells from days 1-6 and day 9, respectively; and lanes 16-21 are *hiwi*-containing vector-transfected KG1 cells from days 1-6 and day 9, respectively. Figure 5B is PCR analysis of the samples in Figure 5A performed without reverse transcription of cellular RNA. Figure 5C is RT-PCR analysis of a GAPD internal control.

Figures 6A through 6L are FACS analyses of apoptosis in KG1 cells (Figures 6A, 6D, 6G and 6J), pCIneo vector-transfected KG1 cells (Figures 6B, 6E, 6H and 6K) and *hiwi*-containing pCIneo vector-transfected KG1 cells (Figures 6C, 6F, 6I and 6L). Figures 6A-C are from cells incubated for 2 hours; Figure 6D-F are from cells incubated for 8 hours; Figure 6G-I are for cells incubated for 12 hours; and Figure 6J-L are for cells incubated for 32 hours.

Figures 7A and 7B are photographs of ethidium bromide stained gel electrophoretic analyses of DNA fragments produced by RT-PCR analysis of RNA isolated from human fetal tissues (Figure 7A) and human adult tissues (Figure 7B) for *hiwi* RNA.

Figures 8A and 8B are photographs of ethidium bromide stained gel electrophoretic analyses of DNA fragments produced by RT-PCR analysis of RNA isolated from human CD34$^+$ bone marrow cells. In Figure 8A, lane 1 is a 123 bp size marker; lane 2 is the fragment produced on day 0 from CD34$^+$ cells; lane 3 is the fragment produced on day 0 from CD34$^-$ cells; lane 4 is the fragment produced from day 1 culture sample; lane 5 is the fragment produced from day 3 culture sample; lane

6 is the fragment produced from day 5 culture sample; lane 7 is the fragment produced from day 7 culture sample; lane 8 is the fragment produced from day 10 culture sample; lane 9 is the fragment produced from day 14 culture sample; lane 10 is a negative control (water); lane 11 is a positive control (human testis). Figure 8B is RT-PCR analysis of a β_2 microglobulin internal control.

Figure 9 is a photograph of a human metaphase chromosome preparation hybridized with a fluorescence-labeled human *hiwi* gene probe.

Figure 10 shows flow cytometric analysis of mock-transfected, vector-transfected and *hiwi*-containing vector transfected KG1 cells.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

The terms "human *hiwi* gene product" and "human *hiwi* gene-encoded protein" as used herein refer to proteins consisting essentially of, and having substantially the same biological activity as, the protein encoded by the amino acid depicted in Figure 1 (SEQ ID No.: 2). This definition is intended to encompass natural allelic variations in the disclosed human *hiwi* gene product. Cloned nucleic acid provided by the present invention may encode *hiwi* gene product of any mammalian species of origin, but preferably the nucleic acid provided by the invention encodes *hiwi* gene product of human origin.

The nucleic acids provided by the invention comprise DNA or RNA having a nucleotide sequence encoding a human *hiwi* gene product. Specific embodiments of said nucleic acid is depicted in Figure 1 (SEQ ID No.: 1), and include any nucleotide sequence encoding a *hiwi* gene product having an amino acid sequence as depicted in Figure 1 (SEQ ID No.: 2). Nucleic hybridization probes as provided by the invention comprise any portion of a nucleic acid of the invention effective in nucleic acid hybridization under stringency conditions sufficient for specific hybridization. Mixtures of such nucleic acid hybridization probes are also within the scope of this embodiment of the invention. Nucleic acid probes as provided herein are useful for isolating mammalian species analogues of the specific embodiments of the nucleic acids provided by the invention. Nucleic acid probes as provided herein are also useful for detecting human *hiwi* gene expression in cells and tissues using techniques well-known in the art, including but not limited to Northern blot hybridization, *in situ* hybridization and Southern hybridization to reverse transcriptase - polymerase chain

reaction product DNAs. The probes provided by the present invention, including

oligonucleotides probes derived therefrom, are also useful for Southern hybridization

of mammalian, preferably human, genomic DNA for screening for restriction

fragment length polymorphism (RFLP) associated with certain genetic disorders and

5   diseases related to differential human *hiwi* gene expression.

        The production of proteins such as human *hiwi* gene product from cloned

genes by genetic engineering means is well known in this art. The discussion that

follows is accordingly intended as an overview of this field, and is not intended to

reflect the full state of the art.

10   Nucleic acid encoding a human *hiwi* gene product may be obtained, in view of

the instant disclosure, by chemical synthesis, by screening reverse transcripts of

mRNA from appropriate cells or cell line cultures, by screening genomic libraries

from appropriate cells, or by combinations of these procedures, in accordance with

known procedures as illustrated below. Additionally, sequences of a human *hiwi* gene

15   product can be obtained from human genomic DNA that has been determined and

assembled in a database or other searchable compilation, using search programs

known in the art and the sequences of the human *hiwi* gene product disclosed herein.

Screening of mRNA or genomic DNA may be carried out with oligonucleotide probes

generated from the nucleic acid sequence information from human *hiwi* gene encoding

20   nucleic acid as disclosed herein. Probes may be labeled with a detectable group such

as a fluorescent group, a radioactive atom or a chemiluminescent group in accordance

with known procedures and used in conventional hybridization assays, as described in

greater detail in the Examples below. In the alternative, human *hiwi* gene-encoding

nucleic acid sequences may be obtained by use of the polymerase chain reaction

25   (PCR) procedure, using PCR oligonucleotide primers corresponding to nucleic acid

sequence information derived from a human *hiwi* gene as provided herein. *See* U.S.

Patent Nos. 4,683,195 to Mullis *et al.* and 4,683,202 to Mullis.

        Nucleic acid and oligonucleotide probes as provided by the present invention

are useful as diagnostic tools for probing human *hiwi* gene expression in tissues of

30   humans and other animals. For example, tissues are probed *in situ* with

oligonucleotide probes carrying detectable groups by conventional autoradiographic

or other detection techniques, to investigate native expression of the *hiwi* gene product

or pathological conditions relating thereto. Further, chromosomes can be probed to

investigate the presence or absence of the corresponding human *hiwi* gene, and

potential pathological conditions related thereto.

Human *hiwi* gene product may be synthesized in host cells transformed with a recombinant expression construct comprising a nucleic acid encoding said gene and comprising genomic DNA or cDNA. Such recombinant expression constructs can also be comprised of a vector that is a replicable DNA construct. Vectors are used herein either to amplify DNA encoding a human *hiwi* gene product and/or to express DNA encoding a human *hiwi* gene. For the purposes of this invention, a recombinant expression construct is a replicable DNA construct in which a nucleic acid encoding a human *hiwi* gene is operably linked to suitable control sequences capable of effecting the expression of the *hiwi* gene product in a suitable host.

The need for such control sequences will vary depending upon the host selected and the transformation method chosen. Generally, control sequences include a transcriptional promoter, an optional operator or enhancer sequence to control transcription, a sequence encoding suitable mRNA ribosomal binding sites, and sequences that control the termination of transcription and translation. Amplification vectors do not require expression control domains. All that is needed is the ability to replicate in a host, usually conferred by an origin of replication, and a selection gene to facilitate recognition of transformants. *See,* Sambrook *et al.,* 2001, Molecular Cloning: A Laboratory Manual, 3rd ed. (Cold Spring Harbor Press: New York).

Vectors useful for practicing the present invention include plasmids, viruses (including phage and mammalian DNA and RNA viruses, particularly adenovirus, adeno-associated virus, lentivirus, and retroviruses), and integratable DNA fragments (*i.e.,* fragments integratable into the host genome by homologous recombination). The vector can replicate the gene of interest and function independently of the host genome, or can, in some instances, integrate into the genome itself. Suitable vectors will contain replicon and control sequences which are derived from species compatible with the intended expression host. A preferred vector is pCIneo (obtained from Promega, Madison, WI). In preferred embodiments, the recombinant expression construct is an inducible construct wherein expression of the *hiwi* gene product can be induced in the cell by an effector molecule. The term "inducible" is intended to encompass vectors wherein expression of the *hiwi* gene product is activated, in either transcription or translation by an effector molecule, most preferably a small molecule or metabolite, wherein induction of *hiwi* gene expression is achieved by contacting the cell with the effector molecule. Examples of effector molecules and inducible

expression include but are not limited to glucocorticoids and recombinant expression constructs having *hiwi* gene expression mediated by a glucocorticoid-responsive promoter; heavy metal ions such as cadmium and recombinant expression constructs having *hiwi* gene expression mediated by a metallothionine promoter; and isopropylthiogalactoside (IPTG) and recombinant expression constructs having *hiwi* gene expression mediated by a promoter comprising all or a functional portion of the bacterial *lac* operon, and further encoding a constitutively expressing a *lac* repressor protein or mammalian or genetically-engineered homolog thereof. *See* Labow *et al.*, 1990, *Molec. Cell. Biol.* 10: 3343-3356.

The recombinant expression constructs of the present invention may also be useful in gene therapy. Cloned genes of the present invention, or fragments thereof, may also be used in gene therapy carried out homologous recombination or site-directed mutagenesis. *See generally* Thomas & Capecchi, 1987, Cell 51: 503-512; Bertling, 1987, Bioscience Reports 7: 107-112; Smithies *et al.*, 1985, Nature 317: 230-234.

Transformed host cells are cells that have been transformed or transfected with recombinant expression constructs made using recombinant DNA techniques and comprising nucleic acid encoding a human *hiwi* gene product. Cultures of cells derived from multicellular organisms are a desirable host for recombinant human *hiwi* gene product synthesis. In principal, any higher eukaryotic cell culture is useful, whether from vertebrate or invertebrate culture. However, mammalian cells are preferred, as illustrated in the Examples, and particularly mammalian cells, most preferably human cells, that do not express an endogenous *hiwi* gene. Propagation of such cells in cell culture has become a routine procedure. *See* Tissue Culture, Academic Press, Kruse & Patterson, editors (1973). Examples of useful host cell lines are human KG1 leukemia cells, human embryonic kidney (HEK) 293 cells, HeLa cells, Chinese hamster ovary (CHO) cell lines, mouse Ltk⁻ cell lines, COS-7, CV1, BHK, and MDCK cell lines. Preferred host cells are HEK293 cells, COS-7 cells (Gluzman, 1981, *Cell* 23: 175-182) and Ltk⁻ cells. Transformed host cells may express the human *hiwi* gene product, but host cells transformed for purposes of cloning or amplifying nucleic acid hybridization probe DNA need not express the human *hiwi* gene. The human *hiwi* gene product of the invention can be located in the host cell cytosol. Accordingly, the invention provides preparations of cell cytosolic fractions comprising the human *hiwi* gene product of the invention, as well as

purified, homogeneous preparations of the human *hiwi* gene product itself. *See,* Sambrook *et al., ibid.* The human *hiwi* gene product of the invention can be located in the host cell nucleus. Accordingly, the invention provides preparations of cell nuclear fractions comprising the human *hiwi* gene product of the invention, as well as purified, homogeneous preparations of the human *hiwi* gene product itself. The human *hiwi* gene product of the invention may also be located in or associated with membranes from the host cell. Therefore, the invention provides preparations of said cell membranes comprising the human *hiwi* gene product of the invention. *See,* Sambrook *et al., ibid.*

The invention provides homogeneous compositions of human *hiwi* gene product produced by transformed eukaryotic cells as provided herein. Each such homogeneous composition is intended to be comprised of a human *hiwi* gene product that comprises at least 75%, more preferably at least 80%, and most preferably at least 90% of the protein in such a homogenous composition; in said homogeneous preparations, individual contaminating protein species are expected to comprise less than 5%, more preferably less than 2% and most preferably less than 1% of the preparation. The invention also provides membrane and cytosolic preparations from cells expressing human *hiwi* gene product as the result of transformation with a recombinant expression construct, as described herein.

Human *hiwi* gene product preparations as provided herewith are useful for identifying compounds that interfere with expression of the *hiwi* gene or activity of the *hiwi* gene product. A compound identified in a screen may be useful for treating various conditions associated with effects of unregulated human *hiwi* gene product activity as a result of endogenous or exogenous over- or underexpression. The present invention provides a pharmaceutical composition comprising the compound in admixture with a pharmaceutically acceptable carrier. In a preferred embodiment, a therapeutically effective amount of the pharmaceutical composition is administered to a patient with a condition associated with unregulated human *hiwi* gene product.

The invention also provides methods for identifying compounds that induce or increase *hiwi* gene expression in mammalian cells, preferably leukemia cells or hematopoietic stem cells and most preferably human leukemia cells and human hematopoietic stem cells, especially CD34$^+$ human hematopoietic stem cells. In these embodiments, the method comprises the steps of culturing a mammalian, most preferably a human cell, most preferably a leukemia cell or hematopoietic stem cell

under conditions wherein the cell does not express the *hiwi* gene or expresses an amount of the *hiwi* gene product insufficient to repress cell proliferation. The method further comprises the steps of contacting the cell with a test compound for a time period, and assaying the cells at intervals during the time period for *hiwi* gene expression and cell proliferation or apoptosis. Compounds that induce *hiwi* gene expression, and concomitantly decrease cell proliferation or increase the percentage of cells undergoing apoptosis are identified thereby.

The method also provides compounds identified by these methods. In preferred embodiments, the compounds induce both hiwi gene expression and quiescence in human hematopoietic stems cells, or induce hiwi expression and inhibit proliferation or promote apoptosis or both in human leukemia cells. Pharmaceutical compositions prepared from such compounds identified by the methods of the invention are also beneficially provided.

Pharmaceutical compositions of the present invention can be manufactured in a manner that is itself known, *e.g.*, by means of a conventional mixing, dissolving, granulating, dragee-making, levigating, emulsifying, encapsulating, entrapping or lyophilizing processes. Pharmaceutical compositions of the compounds of the present invention can be formulated and administered through a variety of means, including systemic, localized, or topical administration. Techniques for formulation and administration can be found in "Remington's Pharmaceutical Sciences," Mack Publishing Co., Easton, PA. Pharmaceutical compositions for use in accordance with the present invention thus can be formulated in conventional manner using one or more physiologically acceptable carriers comprising excipients and auxiliaries that facilitate processing of the active compounds into preparations that can be used pharmaceutically. Proper formulation is dependent upon the route of administration chosen.

Non-toxic pharmaceutical salts include salts of acids such as hydrochloric, phosphoric, hydrobromic, sulfuric, sulfinic, formic, toluenesulfonic, methanesulfonic, nitic, benzoic, citric, tartaric, maleic, hydroiodic, alkanoic such as acetic, HOOC-$(CH_2)_n$-CH_3 where n is 0-4, and the like. Non-toxic pharmaceutical base addition salts include salts of bases such as sodium, potassium, calcium, ammonium, and the like. Those skilled in the art will recognize a wide variety of non-toxic pharmaceutically acceptable addition salts.

For injection, the compounds of the invention can be formulated in appropriate

aqueous solutions, such as physiologically compatible buffers such as Hanks's solution, Ringer's solution, or physiological saline buffer. For transmucosal and transcutaneous administration, penetrants appropriate to the barrier to be permeated are used in the formulation. Such penetrants are generally known in the art.

For oral administration, the compounds can be formulated readily by combining the active compounds with pharmaceutically acceptable carriers well known in the art. Such carriers enable the compounds of the invention to be formulated as tablets, pills, dragees, capsules, liquids, gels, syrups, slurries, suspensions and the like, for oral ingestion by a patient to be treated. Pharmaceutical preparations for oral use can be obtained with solid excipient, optionally grinding a resulting mixture, and processing the mixture of granules, after adding suitable auxiliaries, if desired, to obtain tablets or dragee cores. Suitable excipients are, in particular, fillers such as sugars, including lactose, sucrose, mannitol, or sorbitol; cellulose preparations such as, for example, maize starch, wheat starch, rice starch, potato starch, gelatin, gum tragacanth, methyl cellulose, hydroxypropylmethylcellulose, sodium carboxymethylcellulose, and/or polyvinylpyrrolidone (PVP). If desired, disintegrating agents can be added, such as the cross-linked polyvinyl pyrrolidone, agar, or alginic acid or a salt thereof such as sodium alginate.

Dragee cores are provided with suitable coatings. For this purpose, concentrated sugar solutions can be used, which can optionally contain gum arabic, talc, polyvinyl pyrrolidone, carbopol gel, polyethylene glycol, and/or titanium dioxide, lacquer solutions, and suitable organic solvents or solvent mixtures. Dyestuffs or pigments can be added to the tablets or dragee coatings for identification or to characterize different combinations of active compound doses.

Pharmaceutical preparations that can be used orally include push-fit capsules made of gelatin, as well as soft, sealed capsules made of gelatin and a plasticizer, such as glycerol or sorbitol. The push-fit capsules can contain the active compounds in admixture with filler such as lactose, binders such as starches, and/or lubricants such as talc or magnesium stearate and, optionally, stabilizers. In soft capsules, the active compounds can be dissolved or suspended in suitable liquids, such as fatty oils, liquid paraffin, or liquid polyethylene glycols. In addition, stabilizers can be added. All formulations for oral administration should be in dosages suitable for such administration. For buccal administration, the compositions can take the form of

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tablets or lozenges formulated in conventional manner.

For administration by inhalation, the active compounds for use according to the present invention are conveniently delivered in the form of an aerosol spray presentation from pressurized packs or a nebuliser, with the use of a suitable propellant, *e.g.*, dichlorodifluoromethane, trichlorofluoromethane, dichlorotetrafluoroethane, carbon dioxide or other suitable gas. In the case of a pressurized aerosol the dosage unit can be determined by providing a valve to deliver a metered amount. Capsules and cartridges of *e.g.*, gelatin for use in an inhaler or insufflator can be formulated containing a powder mix of the active compound and a suitable powder base such as lactose or starch.

The active compounds can be formulated for parenteral administration by injection, *e.g.*, by bolus injection or continuous infusion. Formulations for injection can be presented in unit dosage form, *e.g.*, in ampoules or in multi-dose containers, with an added preservative. The compositions can take such forms as suspensions, solutions or emulsions in oily or aqueous vehicles, and can contain formulatory agents such as suspending, stabilizing and/or dispersing agents.

Pharmaceutical formulations for parenteral administration include aqueous solutions of the active compounds in water-soluble form. Additionally, suspensions of the active compounds can be prepared as appropriate oily injection suspensions. Suitable lipophilic solvents or vehicles include fatty oils such as sesame oil, or synthetic fatty acid esters, such as ethyl oleate or triglycerides, or liposomes. Aqueous injection suspensions can contain substances that increase the viscosity of the suspension, such as sodium carboxymethyl cellulose, sorbitol, or dextran. Optionally, the suspension can also contain suitable stabilizers or agents that increase the solubility of the compounds to allow for the preparation of highly concentrated solutions. Alternatively, the active compounds can be in powder form for constitution with a suitable vehicle, *e.g.*, sterile pyrogen-free water, before use. The active compounds can also be formulated in rectal compositions such as suppositories or retention enemas, *e.g.*, containing conventional suppository bases such as cocoa butter or other glycerides.

In addition to the formulations described previously, the active compounds can also be formulated as a depot preparation. Such long acting formulations can be administered by implantation (for example subcutaneously or intramuscularly) or by intramuscular injection. Thus, for example, the active compounds can be formulated

with suitable polymeric or hydrophobic materials (for example as an emulsion in an acceptable oil) or ion exchange resins, or as sparingly soluble derivatives, for example, as a sparingly soluble salt.

The pharmaceutical compositions also can comprise suitable solid or gel phase carriers or excipients. Examples of such carriers or excipients include but are not limited to calcium carbonate, calcium phosphate, various sugars, starches, cellulose derivatives, gelatin, and polymers such as polyethylene glycols.

The active compounds of the invention can be provided as salts with pharmaceutically compatible counterions. Pharmaceutically compatible salts can be formed with many acids, including but not limited to hydrochloric, sulfuric, acetic, lactic, tartaric, malic, succinic, phosphoric, hydrobromic, sulfinic, formic, toluenesulfonic, methanesulfonic, nitic, benzoic, citric, tartaric, maleic, hydroiodic, alkanoic such as acetic, $HOOC\text{-}(CH_2)_n\text{-}CH_3$ where n is 0-4, and the like. Salts tend to be more soluble in aqueous or other protonic solvents that are the corresponding free base forms. Non-toxic pharmaceutical base addition salts include salts of bases such as sodium, potassium, calcium, ammonium, and the like. Those skilled in the art will recognize a wide variety of non-toxic pharmaceutically acceptable addition salts.

The mode of administration can be selected to maximize delivery to a desired target site in the body. Suitable routes of administration can, for example, include oral, rectal, transmucosal, transcutaneous, or intestinal administration; parenteral delivery, including intramuscular, subcutaneous, intramedullary injections, as well as intrathecal, direct intraventricular, intravenous, intraperitoneal, intranasal, or intraocular injections. Alternatively, one can administer the compound in a local rather than systemic manner, for example, *via* injection of the compound directly into a specific tissue, often in a depot or sustained release formulation.

Pharmaceutical compositions suitable for use in the present invention include compositions wherein the active ingredients are contained in an effective amount to achieve its intended purpose. More specifically, a therapeutically effective amount means an amount effective to prevent development of or to alleviate the existing symptoms of the subject being treated. Determination of the effective amounts is well within the capability of those skilled in the art, especially in light of the detailed disclosure provided herein.

For any compound used in the method of the invention, the therapeutically effective dose can be estimated initially from cell culture assays, as disclosed herein.

For example, a dose can be formulated in animal models to achieve a circulating concentration range that includes the EC50 (effective dose for 50% increase) as determined in cell culture, *i.e.*, the concentration of the test compound which achieves a half-maximal inhibition of tumor cell growth *in vitro*. Such information can be used to more accurately determine useful doses in humans.

It will be understood, however, that the specific dose level for any particular patient will depend upon a variety of factors including the activity of the specific compound employed, the age, body weight, general health, sex, diet, time of administration, route of administration, and rate of excretion, drug combination, the severity of the particular disease undergoing therapy and the judgment of the prescribing physician.

For administration to non-human animals, the drug or a pharmaceutical composition containing the drug may also be added to the animal feed or drinking water. It will be convenient to formulate animal feed and drinking water products with a predetermined dose of the drug so that the animal takes in an appropriate quantity of the drug along with its diet. It will also be convenient to add a premix containing the drug to the feed or drinking water approximately immediately prior to consumption by the animal.

Toxicity and therapeutic efficacy of such compounds can be determined by standard pharmaceutical procedures in cell cultures or experimental animals, *e.g.*, for determining the LD50 (the dose lethal to 50% of the population) and the ED50 (the dose therapeutically effective in 50% of the population). The dose ratio between toxic and therapeutic effects is the therapeutic index and it can be expressed as the ratio between LD50 and ED50. Compounds that exhibit high therapeutic indices are preferred. The data obtained from these cell culture assays and animal studies can be used in formulating a range of dosage for use in humans. The dosage of such compounds lies preferably within a range of circulating concentrations that include the ED50 with little or no toxicity. The dosage can vary within this range depending upon the dosage form employed and the route of administration utilized. The exact formulation, route of administration and dosage can be chosen by the individual physician in view of the patient's condition. (*See, e.g.* Fingl *et al.*, 1975, in "The Pharmacological Basis of Therapeutics", Ch.1, p.1).The recombinant expression constructs of the present invention are useful in molecular biology to transform cells that do not ordinarily express a hiwi protein to thereafter express this protein. Such

cells are useful as intermediates for making cell membrane, nuclear or cytosolic preparations useful, *inter alia*, drug screening. The recombinant expression constructs of the present invention thus provide a method for screening potentially useful drugs at advantageously lower cost than conventional animal screening protocols. While not completely eliminating the need for ultimate *in vivo* activity and toxicology assays, the constructs and cultures of the invention provide an important first screening step for the vast number of potentially useful drugs synthesized, discovered or extracted from natural sources each year.

The invention also provides antibodies that are immunologically reactive to the human *hiwi* gene product or epitopes thereof provided by the invention. The antibodies provided by the invention may be raised, using methods well known in the art, in animals by inoculation with cells that express a human *hiwi* gene product or epitopes thereof, cell membranes from such cells, whether crude membrane preparations or membranes purified using methods well known in the art, cytosolic preparations, or purified preparations of proteins, including fusion proteins, particularly fusion proteins comprising epitopes of the human *hiwi* gene product of the invention fused to heterologous proteins and expressed using genetic engineering means in bacterial, yeast or eukaryotic cells, said proteins being isolated from such cells to varying degrees of homogeneity using conventional biochemical methods. Synthetic peptides made using established synthetic methods *in vitro* and optionally conjugated with heterologous sequences of amino acids, are also encompassed in these methods to produce the antibodies of the invention. Animals that are useful for such inoculations include individuals from species comprising cows, sheep, pigs, chickens, mice, rats, rabbits, hamsters, goats and primates. Preferred animals for inoculation are rodents (including mice, rats, hamsters) and rabbits. The most preferred animal is the mouse.

Cells that can be used for such inoculations, or for any of the other means used in the invention, include any cell line which naturally expresses the human *hiwi* gene product provided by the invention, or more preferably any cell or cell line that expresses the human *hiwi* gene product of the invention, or any epitope thereof, as a result of molecular or genetic engineering, or that has been treated to increase the expression of an endogenous or heterologous human *hiwi* gene product by physical, biochemical or genetic means. Preferred cells are mammalian cells, most preferably cells syngeneic with a rodent, most preferably a mouse host, that have been

transformed with a recombinant expression construct of the invention encoding a human *hiwi* gene product, and that express the gene product therefrom.

The present invention also provides monoclonal antibodies that are immunologically reactive with an epitope derived from a human *hiwi* gene product of the invention, or fragment thereof. Such antibodies are made using methods and techniques well known to those of skill in the art. Monoclonal antibodies provided by the present invention are produced by hybridoma cell lines, that are also provided by the invention and that are made by methods well known in the art.

Hybridoma cell lines are made by fusing individual cells of a myeloma cell line with spleen cells derived from animals immunized with cells expressing a human *hiwi* gene product of the invention, as described above. The myeloma cell lines used in the invention include lines derived from myelomas of mice, rats, hamsters, primates and humans. Preferred myeloma cell lines are from mouse, and the most preferred mouse myeloma cell line is P3X63-Ag8.653. The animals from which spleens are obtained after immunization are rats, mice and hamsters, preferably mice, most preferably Balb/c mice. Spleen cells and myeloma cells are fused using a number of methods well known in the art, including but not limited to incubation with inactivated Sendai virus and incubation in the presence of polyethylene glycol (PEG). The most preferred method for cell fusion is incubation in the presence of a solution of 45% (w/v) PEG-1450. Monoclonal antibodies produced by hybridoma cell lines can be harvested from cell culture supernatant fluids from *in vitro* cell growth; alternatively, hybridoma cells can be injected subcutaneously and/or into the peritoneal cavity of an animal, most preferably a mouse, and the monoclonal antibodies obtained from blood and/or ascites fluid.

Monoclonal antibodies provided by the present invention are also produced by recombinant genetic methods well known to those of skill in the art, and the present invention encompasses antibodies made by such methods that are immunologically reactive with an epitope of a human *hiwi* gene product of the invention. The present invention also encompasses fragments, including but not limited to F(ab) and F(ab)$_2$ fragments, of such antibody. Fragments are produced by any number of methods, including but not limited to proteolytic or chemical cleavage, chemical synthesis or preparation of such fragments by means of genetic engineering technology. The present invention also encompasses single-chain antibodies that are immunologically reactive with an epitope of a human *hiwi* gene product, made by methods known to

those of skill in the art.

The present invention also encompasses one or a plurality epitope of a human *hiwi* gene product of the invention, comprised of sequences and/or a conformation of sequences present in the molecule. This epitope may be naturally occurring, or may be the result of chemical or proteolytic cleavage of a human *hiwi* gene product and isolation of an epitope-containing peptide or may be obtained by chemical or *in vitro* synthesis of an epitope-containing peptide using methods well known to those skilled in the art. The present invention also encompasses epitope peptides produced as a result of genetic engineering technology and synthesized by genetically engineered prokaryotic or eukaryotic cells.

The invention also includes chimeric antibodies, comprised of light chain and heavy chain peptides immunologically reactive to a human *hiwi* gene product -derived epitope. The chimeric antibodies embodied in the present invention include those that are derived from naturally occurring antibodies as well as chimeric antibodies made by means of genetic engineering technology well known to those of skill in the art.

The invention also provides methods for maintaining or increasing the number of primitive CD34$^+$ hematopoietic stem cells in peripheral blood culture or *in vitro* bone marrow culture. During steady state hematopoiesis, most stem cells are quiescent or cycling extremely slowly (Ando *et al.*, 1993, *Proc. Natl. Acad. Sci. USA* 90:9571-9575; Abkowitz *et al.*, 1995, *Proc. Natl. Acad. Sci. USA* 92:2031-2035; Abkowitz *et al.*, 1996, *Nature Med.* 2:190-197; Uchida *et al.*, 1996, *Blood* 88:1297-1305; Morrison *et al.*, 1997, *Proc. Natl. Acad. Sci. USA* 94:1908-1913; Cheshier *et al.*, 1999, *Proc. Natl. Acad. Sci. USA* 96:3120-3125). Stem cell quiescence may be a passive process involving the absence of proliferation or an active process that occurs as a consequence of a variety of negative inhibitors of hematopoiesis (Akazawa *et al.*, 1992, *J. Biol. Chem.* 267:21879-21885; Bradford *et al.*, 1997, *Exp Hematol.* 25:445-453; Postigo *et al.*, 1997, *EMBO J.* 5:3935-3943). The observation that *hiwi* expression is associated with diminished proliferation of an immortalized leukemia cell line suggests that the expression of this gene might play a role in maintenance of stem cell quiescence or down regulation of stem cell or progenitor cell cycling. This is somewhat surprising since it has been reported that *piwi* causes cellular division within a *Drosphila* based model (Cox *et al.*, 2000, *Development* 127:503-514). In *Drosophila, piwi* is expressed both in the terminal filament cells and the germ line. The *piwi* gene in the terminal filament functions to affect stem cell self-replication

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while *piwi* expression in the germ line does not appear to be required for GSC self-replication (Cox *et al.*, 1998, *Genes Dev.* $\underline{12}$:3715-3727). Many of the regulatory signals that control stem cell development are dependent upon cellular interactions between marrow stroma and hematopoietic stem cells (Dexter *et al.*, 1977, *ibid.*; .Wineman *et al.*, 1996, *ibid.*). Although *hiwi* was expressed in marrow CD34$^+$ cells, it was not expressed by marrow stroma or marrow mesenchymal stem cells that are capable of differentiating into not only marrow stroma but also other componemts of the hematopoietic niche such as adipocytes, osteoblasts, tenoblasts and cartilage forming cells (Pittenger *et al.*, 1999, *ibid.*). The data disclosed herein indicate that the potential role of *hiwi* in human stem cell development is quite different from that which occurs in the *Drosophila* model, and that the *hiwi* present in CD34$^+$ cells may play a role as an intrinsic regulator of stem cell self-replication.

Thus, the invention provides a method for increasing the percentage of primitive CD34$^+$ hematopoietic stem cells in an *in vitro* bone marrow culture or peripheral blood culture. In one embodiments, the inventive method comprises the step of culturing the bone marrow or peripheral blood culture cells in the presence of a *hiwi* gene inducing compound of the invention. In alternative embodiments, the inventive method comprises the step of introducing into the cell a recombinant expression construct of the invention encoding a hiwi gene, most preferably a human *hiwi* gene. In preferred embodiments, *hiwi* gene expression mediated by the recombinant expression construct is inducible gene expression.

The Examples that follow are illustrative of specific embodiments of the invention, and various uses thereof. They set forth for explanatory purposes only, and are not to be taken as limiting the invention.

EXAMPLE 1

Cell culture preparations and assays

<u>Isolation of human and baboon CD34$^+$ cells</u>

CD34$^+$ bone marrow cells contain hematopoietic stem and early progenitor cells, in contrast to CD34$^-$ cells, which are predominantly composed of more differentiated precursor cells. Adult human bone marrow (BM) samples (15 to 30 mL) were aspirated from the posterior iliac crests of normal donors after informed consent was obtained according to established guidelines. Heparinized marrow

aspirates were diluted with Ca^{++}- and Mg^{++}-free Dulbecco's phosphate-buffered saline (DPBS) (BioWhittaker, Walkersville, MD). Diluted marrow was then underlaid with Ficoll-Paque (Pharmacia AB, Uppsala, Sweden), and centrifuged at 800x g for 30 minutes at 20°C. The mononuclear cell fraction was collected and $CD34^+$ cells were

5   immunomagnetically enriched using the MACS CD34 Isolation Kit (Miltenyi Biotec, Auburn, CA) according to the manufacturer's instructions. Briefly, cells were incubated with hapten-labeled anti-CD34 antibody (QBEND-10, Becton Dickinson) in the presence of blocking reagent, human IgG (Bayer Corporation Elkhart, IN), and then with anti-hapten coupled to MACS microbeads. Labeled cells were filtered

10   through a 30 μm nylon mesh and separated using a high-gradient magnetic separation column. Magnetically retained cells were eluted and stained with $CD34^+$ specific monoclonal antibodies (MoAb) and analyzed using flow cytometric methods. The flow-through population was identified as $CD34^-$ cells. The purity of the $CD34^+$ population was routinely greater than 90%.

15   BM aspirates were obtained from the humeric and iliac crests of juvenile baboons (*Papio anubis*) after ketamine (10 mg/kg) and xylazine (1 mg/kg) anesthesia. Heparinized marrow was diluted 1:15 in phosphate-buffered saline (PBS) and the mononuclear cell fraction obtained by centrifugation over 60% Percoll (Pharmacia AB) at 500x g for 30 minutes at 20°C. Antihuman CD34 monoclonal antibody K6.1

20   (a gift from the Naval Medical Research Institute, Bethesda, MD), a murine IgG_{2a} that cross-reacts with baboon CD34 antigen, was used to select the $CD34^+$ fraction of marrow cells (Davis *et al.*, 1995, *Blood* 85: 1751-1761) This isolated mononuclear cell fraction was suspended in PBS containing 0.2% bovine serum albumin (BSA; Sigma Chemical Co, St Louis, MO) and stained first with biotin-conjugated K6.1 (20

25   μg/mL), washed, and labeled with Miltenyi streptavidin-conjugated iron microbeads (Miltenyi Biotec) and selected as described above according to manufacturer's instructions.

Flow cytometric analysis and sorting

30   Isolated human $CD34^+$ cells were further fractionated based upon the expression of CD38 antigen (Terstappen *et al.*, 1991, *Blood* 77: 1218-1227) Nonspecific staining was blocked using 0.1% heat-inactivated human gamma globulin (Bayer). Cells were stained with anti-CD34 MoAb conjugated to fluorescein isothiocyanate (FITC) (Becton Dickinson) and anti CD38 labeled with phycoerythrin

(PE) (Becton Dickinson). Control cells were incubated with fluorochrome-conjugated isotype-matched IgG1-FITC (Becton Dickinson) and IgG1-PE (Becton Dickinson). Immediately prior to sorting, propidium iodide (PI) at a concentration of 1 μg/mL was added to identify and exclude nonviable cells. Cells were sorted and analyzed on a FACSVantage cell sorter (Becton Dickinson). FITC, PE and PI were excited at a wavelength of 488 nm using an argon ion laser. The CD34$^+$ cells were sorted into CD38$^-$, CD38^{lo}, and CD38^{hi} subpopulations. Positive fluorescence for each of the markers was established as fluorescence greater than 99% of isotype-matched irrelevant murine IgG$_1$ controls. Cell aggregates or debris were excluded by forward and 90° light scatter. All staining for analysis and sorting was done in the presence of 0.2% BSA in PBS on ice.

Stroma-free expansion cultures

To promote differentiation of human CD34$^+$ cells, a stroma-free suspension culture was established as described by Bazil et al. (1995, Blood 86: 502-511) Tissue culture dishes (35mm; Corning., Corning, NY) were seeded with 1x10^6 CD34$^+$ cells/ well in 3 mL of Iscove modified Dulbecco medium (IMDM) (BioWhittaker) containing 10% fetal bovine serum (FBS; Hyclone, Logan, UT). Cultures were placed at 37°C in 100% humidified atmosphere of 5% CO$_2$ in air. Cultures received a combination of recombinant cytokines at initiation of the cultures and at 72- to 96-hour intervals; these cytokines included stem cell factor (SCF), interleukin (IL)-3, and granulocyte-colony stimulating factor (G-CSF) all at a concentration of 100 ng/mL (R&D Systems, Minneapolis, MN). Cultures were maintained at a cell concentration of 5 x10^5 to 2 x10^6 viable cells/mL.

Leukemia cell lines

The following leukemia cell lines were used: TF-1 lymphoblast cell line; Jurkat, a T lymphocyte cell line; CEM, an acute T lymphoblast cell line; BV-173, a B cell precursor cell line; K-562, a chronic myeloid cell line; KG1 and KG1a, acute myeloid cell lines (all obtained through the American Type Culture Collection, Manassas, VA) and SUPB13, an acute B lymphoblastic cell line (a gift from Steve Smith at the University of Chicago, Chicago, IL). The lines were maintained in RPMI 1640 (BioWhittaker) supplemented with 10% heat-inactivated FBS (except for KG1 and KG1a which required the presence of 20% FBS), 2 mM L-glutamine, 100 U/mL

penicillin, 1 mg/mL streptomycin (BioWhittaker). In addition, granulocyte/ macrophage CSF (GM-CSF; 5ng/mL) (PeproTech, Rocky Hill, NJ) was added to TF-1 culture. Cell density was maintained at $1x10^5$ to $1x10^6$ viable cells/mL.

5   Mesenchymal stem cells

Mesenchymal stem cells were isolated and expanded from normal human marrow aspirates as described by Pittenger et al. (1999, Science 284:143-147). These purified cells were provided commercially by Osiris Therapeutics (Baltimore, MD).

10   Stromal cells

Bone marrow stroma was grown by seeding 1 x 10^6 low density bone marrow cells/ 162 cm^2 (Corning) flask in low-glucose Dulbecco modified Eagle medium (DMEM; Mediatech, Herndon, VA) supplemented with 10% heat-inactivated FBS and 2 mM L-glutamine, 100 U/mL penicillin, 1 mg/mL streptomycin (Dexter et al.,
15   1977, J Cell Physiol. 91:335-344; Wineman et al., 1996, Blood 87:4082-4090). Adherent cells were split at confluency and the nonadherent cells were discarded. Adherent cells were passaged 4 times and were then termed marrow stroma (Dexter et al., 1977, ibid.; Wineman et al., 1996, ibid.).

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EXAMPLE 2
Cloning of Human Homolog of *Drosophila* piwi Gene (hiwi)

A complementary DNA (cDNA) clone of the human homolog (*hiwi*) of the *Drosophila melanogaster* piwi gene was prepared as follows.
25   *Hiwi* gene expression was detected in CD34$^+$ cells using a semi-quantitative polymerase chain reaction (PCR) assay of reverse-transcribed cellular RNA isolated from immunomagnetically separated CD34$^+$ and CD34$^-$ cells from non-human primates (*Papio anubis*), and from humans. Total RNA was isolated from FACS-separated hematopoietic, stromal, and mesenchymal cells cultured as described in
30   Example 1 using Trizol reagent (Life Technologies, Gaithersburg, MD) according to manufacturer's instructions. Cells were pelleted and then resuspended in 1mL of Trizol per $5x10^6$ cells by repeated pipetting. The cell lysate was then incubated for 5 minutes at room temperature and extracted with 0.2 volumes of chloroform by vortexing for 1 minute. The sample was thereafter centrifuged for 30 minutes at

13,000 rpm (12,000g), 4°C in a microcentrifuge. The RNA was precipitated using 2 volumes isopropanol, mixed and allowed to sit at room temperature for 10 minutes. RNA was centrifuged for 45 minutes at 13,000 rpm (12,000x g). The pelleted RNA was washed with 75% ethanol, briefly dried, and resuspended in RNase-free water or diethyl pyrocarbonate-treated (DEPC; Sigma) water (0.1%). The amount of RNA in the preparation was then quantitated using a DU 650 spectrophotometer (Beckman Instruments, Palo Alto, CA). The RNA was treated with DNase I enzyme (Life Technologies) according to the manufacturer's instructions.

Polymerase chain reaction (RT-PCR) amplification was performed on RNA samples using an RNA PCR Core Kit (PerkinElmer, Foster City, CA), according to the manufacturer's instruction, except that High Fidelity Platinum *Taq* DNA Polymerase (Life Technologies) was substituted for AmpliTaq. Alternatively, a Stratagene ProSTAR First-Strand RT-PCR Kit (Stratagene, La Jolla, CA) was used for amplification. One microgram of total RNA was used for cDNA synthesis using random hexamers to prime first strand synthesis. The synthesized $CD34^+$ cDNA was divided and used for PCR amplification. As a control, duplicate cDNA synthesis reactions were performed for each experiment without the addition of reverse transcriptase. Control PCR amplification reactions were performed using primers for glyceraldehyde phosphate dehydrogenase cDNA (GAPD; forward primer, 5'-GGCTGAGAACGGGAAGCTTGTCAT-3'(SEQ ID NO.: 3); reverse primer, 5'-CAGCCTTCTCCATGGTGGTGAAGA-3' (SEQ ID NO.: 4)) for 1 cycle at 94°C/2 min; 5 cycles at 94°C/10s, 70°C/2min; 5 cycles at 94°C/10s, 68°C/2 min; 25 cycles at 94°C/10s, 66°C/2min, and 1 cycle at 72°C /10min and produced a 142-bp product. β_2 microglobulin primers were also used as an internal control (β_2 microglobulin: forward primer, 5'-CTCGCGCTACTCTCTCTTTC-3 (SEQ ID NO. 5); reverse primer, 5'-CATGTCTCGATCCCACTTAAC-3' (SEQ ID NO. 6)) producing a 329-bp product. PCR amplification primers were designed based on the partial published *hiwi* DNA sequence found in the Genbank database (accession number AF104260). Detection of *hiwi* in $CD34^+$ DNase treated RNA was performed using the primer pair hiwiF269 5'-GAAGCAGCCTGTCTTGGTCAGC-3' (SEQ ID NO. 7) and hiwiR269 5'-GAATCAAAGCTCAAACCCCAGTCTC-3' (SEQ ID NO. 8) producing a 269 bp product.

The semiquantitative RT-PCR assay produced a *hiwi*-specific 269bp DNA fragment 269bp from $CD34^+$ cells; an identically-sized fragment was also found after

RT-PCR amplification of human testis cDNA. This fragment was specific for cDNA prepared from CD34$^+$ cells and was not observed after amplification of CD34$^-$ cell cDNA from either humans or baboons. Direct DNA sequencing of the PCR product was performed using an ABI Prism Dye Terminator Cycle Sequencing Reaction Kit (PerkinElmer) according to the manufacturer's instructions. Additional primers (Integrated DNA Technologies, Coralville, IA) were synthesized based upon analyzed sequence in order to obtain the complete cDNA sequence. This analysis confirmed that the PCR product fragment was a fragment of the *hiwi* cDNA (Figure 1).

To examine whether *hiwi* gene expression was restricted to the most primitive subpopulation of human CD34$^+$ cells, CD34$^+$ cells were sorted according to CD38 expression into 3 subpopulations: CD34$^+$CD38$^-$, CD34$^+$CD38^{lo}, CD34$^+$CD38^{hi} (Figure 2A). Semiquantitative RT-PCR on DNase treated RNA isolated from each population showed that each of the 3 subpopulations expressed *hiwi*. These data indicate the *hiwi* expression is not limited to the most primitive progenitor cell population (Figure 2B).

The full-length human *hiwi* gene was cloned as follows. A primer pair that spanned from amino acid 364 to 524 of the published partial coding sequence was designed and produced a 480-bp fragment that corresponded to the C-terminal end of the protein. After positively identifying the PCR product to be that of *hiwi* through dye terminator cycle sequencing (Sambrook *et al.*, 2001, *ibid.*), primers were designed to amplify the potential full length gene from human testis cDNA by using a 5'RACE cloning methodology that allows the PCR amplification of a given gene of interest by utilizing a small region of a known sequence. Two primer pairs were designed to be employed in 5' Rapid Amplification of cDNA Ends (5' RACE) PCR strategy cloning (Chenchik *et al.*, 1996, *Biotechniques.* 21:526-534):

AKSrev1, reverse primer #1: 5'-CGCTGTATGTGGTCTGGCTTCAGGC-3' (SEQ ID NO. 9) and

AKSrev2, reverse primer #2; 5'-GGGAGAAACACTACCACTTCTCACAGCCTG-3' (SEQ ID NO. 10).

AKSrev2 is located 32 nucleotides upstream from AKSrev1 and serves as a nested internal control for secondary PCR amplification. AKSrev1 and AKSrev2 were also based upon the published partial *hiwi* coding sequence and acted as a nested primer pair for primary and secondary PCR amplification reactions and correspond to base pairs 1391 to 1415 and 1330 to 1359, respectively. Marathon Ready Human Testis cDNA Kit (Clontech Laboratories, Inc., Palo Alto, CA) was used for primary

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amplification, according to manufacturer's instructions. First round PCR amplification consisted of AKSrev1 and AP-1 (5'-CCATCCTAATACGACTCACTATAGGGC-3' (SEQ ID NO. 11), supplied within the cDNA Kit) under the following conditions: 1 cycle at 94°C/2 min; 5 cycles at 94°C/10s, 71°C/2min; 5 cycles at 94°C/10s, 69°C/2 min; 25 cycles at 94°C/10s, 67°C/2min, and 1 cycle at 72°C /10min. A single PCR product was separated on a 1% SeaPlaque Agarose (FMC, Rockland, ME) 1x TAE gel stained with ethidium bromide and purified from the agarose using a Wizard PCR Prep Kit (Promega Corporation, Madison, WI). The isolated PCR fragment was then used in a second round of PCR amplification that utilized the same PCR amplification conditions listed above except that AKSrev2 was substituted for AKSrev1 and AP-2 (5'-ACTCACTATAGGGCTCGAGCGGC-3' (SEQ ID NO. 12) replaced AP-1. All PCR reactions were performed in a PerkinElmer Thermal Cycler 9700 (Perkin Elmer) or Stratagene Robocycler Gradient 96 Thermal Cycler.

After employing the 5' RACE methodology on the human testis cDNA sequence and two rounds of PCR amplification (the second round consisting of a nested PCR amplification) a putative 2.3 kb full length coding sequence was cloned. After several rounds of sequencing through primer walking, an open reading frame was determined, and is shown in Figure 1 (SEQ ID NO. 1). When compared to the Genbank database of non-redundant clones, the human HIWI protein (SEQ ID NO> 2) showed a 52% homology to the *Drosophila* PIWI protein (SEQ ID NO. 13) at the amino acid level (Figure 1).

Primer pairs were then designed to amplify the full length coding sequence from CD34$^+$ hematopoietic cell cDNA through PCR amplification. Based upon a sequence obtained from the human testis clone, primers were designed to amplify a putative full-length cDNA clone,
FLhiwifor1 (forward primer, 5'-ATGATCTTTGGTGTGAACACAAGGCAGAA-3' (SEQ ID NO. 14) and
FLhiwirev1 (reverse primer, 5'-GAGGTAGTAAAGGCGGTTTGACAGTGACAGA-3' (SEQ ID NO. 15).
PCR amplification conditions were as follows: 1 cycle at 94°C/2 min; 5 cycles at 94°C/10s, 72°C/2min; 5 cycles at 94°C/10s, 70°C/2 min; 25 cycles at 94°C/10s, 68°C/2min, and 1 cycle at 72°C /10min. A 2.3 kb band was detected in the CD34$^+$ cDNA sample but not in the no-template control. No bands were detectable in the

absence of reverse transcriptase (-RT) during the cDNA synthesis step. Control PCR amplification with primers specific for glyceraldehyde phosphate dehydrogenase (GAPD) confirmed that the quantity and integrity of the RNA could be PCR amplified. The PCR product was sequenced and the identity was confirmed to be that of the *hiwi* gene by comparing it to the Genbank database.

After obtaining the full-length *hiwi* cDNA, the fragment was then subcloned into the *XhoI/NotI* sites of the pCIneo Mammalian Expression Vector (Promega) and used for expression studies as described below.

EXAMPLE 3

Recombinant Expression of Cloned Human *hiwi* Gene

A human leukemia cell line, KG1 cells were chosen as recipients for recombinant human *hiwi* production due to their lack of expression of the *hiwi* mRNA as demonstrated by RT-PCR (Figure 3). A recombinant expression construct encoding human *hiwi* was introduced into these cells by electroporation. Electroporation of plasmid DNA into KG1 cells was accomplished using a Bio-Rad Gene Pulser II System (Bio-Rad Laboratories, Hercules, CA) under conditions of 300 V and 950 μF using a 0.4 cm gapped cuvette (Bio-rad Laboratories). Fifty micrograms of pCIneo or pCIneo-*hiwi* was electroporated per 1×10^5 KG1 cells, accompanied with 2 μg of linearized vector for each respective condition as well as a mock control that consisted of KG1 cells alone. All cells were washed with and then resuspended in DPBS prior to electroporation. Cells were then centrifuged and washed twice with DPBS and resuspended in IMDM supplemented with 20% heat inactivated FBS, 100 U/mL penicillin, 1 mg/mL streptomycin, and 2 mM L-glutamine and incubated overnight at 37°C in a 100% humidified incubator containing 5% CO_2 in air. After overnight incubation, cells were counted and stained for viability using a 0.4% trypan blue solution (Sigma) on the following day. Cells were then plated at a density of 2×10^4 viable cells per well in a flat bottom 96 well plate (Corning) in IMDM supplemented with 5% heat inactivated FBS, 100 U/mL penicillin, 1 mg/mL streptomycin, and 2 mM L-glutamine. Similar populations of cells (pCIneo vector alone, the pCIneo-hiwi, or cells alone) were incubated in IMDM supplemented with 20% FBS, 100 U/mL penicillin, 1 mg/mL streptomycin, and 2 mM L-glutamine containing 1 mg/mL Geneticin Selective Antibiotic (G418 sulfate; Life Technologies)

for 3 weeks. Several sets of transfected cells were plated under similar conditions for detection of the *hiwi* gene by PCR and for antibiotic selection. G418 resistant colonies were assayed by RT-PCR for expression of human *hiwi* mRNA.

The proliferative capacity of KG1 cells expressing the recombinant human *hiwi* gene was determined using an MTT exclusion assay. In this assay, cell proliferation and survival was measured by cellular uptake of MTT{(3,-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; Sigma-Aldrich),. which measures living cells (Bazil *et al.*, 1995, *ibid.*). Fifty microliters of a 1 mg/mL solution of filter sterilized MTT in DPBS was added to each culture volume of 200 µL containing 2×10^4 viable cells per well and then incubated for 4 hours at 37°C in a 100% humidified incubator, 5%CO_2 in air. Approximately half of the volume was then carefully removed (without disrupting the solubilized complex) and replaced with developing reagent that consisted of 40 mM hydrochloric acid in isopropanol. After thoroughly homogenizing the solubilized complex with the developing reagent, the plate was then read on an ELX-800 ELISA plate reader (Bio-Tek Instruments, Winooski, VT) at wavelength of 570 nm with a reference wavelength of 630 nm as previously described (Sharma *et al.*, 2001, *Blood* 97: 426-434). This procedure was performed on days 1 to 6 and then on day 9.

The results of these assays are shown in Figure 4. As shown in the Figure, the proliferative capacity of mock transfected and pCIneo vector treated cells was similar. KG1 cells transfected with either the empty vector control or the mock transfection control were capable of proliferating normally without the presence of the *hiwi* gene or interference from the empty vector control. However, KG1 cells transfected with the pCIneo-*hiwi* construct showed greatly diminished proliferation. To determine whether the diminished proliferation was accompanied by *hiwi* gene expression in the transfected cells, RT-PCR analysis was performed as described above. These assays, the results of which are shown in Figure 5, demonstrated the presence of the *hiwi* transcript from day 1 (24 hours post transfection) until cultures were terminated (day 9). The declining levels of the expressed gene was likely due to the fact that the cells were not subjected to G418 antibiotic selection during the assay.

To further assess the function and integrity of each construct, populations of cells that had been simultaneously transfected were placed in the presence of 1 mg/mL of active G418 for 3 weeks. After this period, the pCIneo-*hiwi* construct and the pCIneo empty vector contained similar numbers of viable cells. The pCIneo-*hiwi*

construct further continued to express the *hiwi* gene (as determined by PCR). The mock transfected cells were, however, characterized by a high degree of cell death at day 6 (>96%, data not shown). These results demonstrate that both the cytomegalovirus (CMV) promoter and the SV40 promoter (which was driving neomycin phosphotransferase gene) were actively driving transcription of their respective genes and that *hiwi* gene expression was present, but to an unknown level.

The cause of the observed reduced proliferative capacity of KG1 cells that overexpress the *hiwi* gene product was also investigated by examining apoptosis (programmed cell death) of these cells. Apoptosis was evaluated using Annexin V as an indicator of programmed cell death and PI as a measure of cell viability. KG1 cells were transfected under the three separate conditions as previously described. Condition 1 consisted of a mock transfection, condition 2 consisted of an empty vector control transfection, and condition 3 consisted of the vector containing *hiwi*. An additional sample of KG1 cells was also serum starved for 24 hours in IMDM, 2mM L-glutamine, 100U/mL penicillin, and 1mg/mL streptomycin (BioWhittake). Incubation conditions are similar to those described above. Transfected cells were maintained in culture for up to 32 hours and then assayed for programmed cell death by the Annexin V Assay System (Pharmingen, San Diego, CA) according to the manufacturer's instructions. Briefly, cells were washed with DPBS, collected and then resuspended in binding buffer containing Annexin V-FITC and PI at room temperature. Acquisition and analysis of data was performed by FACS on a FACSCalibur (Becton Dickinson) using the CellQuest Analysis Software (Becton Dickinson).

Figures 6 D-F shows the results of such an analysis after an 8 hour incubation, and demonstrates an approximate 4.4 fold increase (9.6%) in the percentage of cells undergoing apoptosis, (positive for Annexin V but negative for PI) when compared to the mock (1.8%) and the empty vector control (2.4%). The degree of apoptosis after 12 hours of incubation is also greater in the *hiwi* overexpressing cells as compared to the control cells. Flow cytometric analysis at 32 hours of culture (Figures 6J-L) exhibited an increase in the number of cells that were PI and Annnexin positive suggesting that the cells had proceeded to a necrotic state. These data indicate that the majority of the cell population containing the *hiwi* gene underwent apoptosis and eventually entered a terminal state of cell death. The rapid progression of apoptosis can be seen at the 8-hour timepoint where a majority of the cells in the *hiwi* containing

population have undergone apoptosis and have started to proceed into the necrotic state. These studies suggest that *hiwi* overexpression causes programmed cell death. Lack of apoptosis in the cells transfected with the *hiwi* gene which were maintained in selective media maybe due to the SV40 promoter, (which drives the transcription of the neomycin phosphotransferase gene), out competing the CMV promoter (that directs the transcription of the *hiwi* gene) because of the selective pressure placed upon the SV40 promoter by the G418 selection. This may account for diminished expression of the *hiwi* gene product while the transcription of the neomycin phosphotransferase gene continues. Emerman *et al* (1984, *Cell* 39:449-467) have reported a state of gene suppression in cells genetically modified with a retrovirus in which one gene is shutdown while the second gene undergoes normal transcription due to promoter competition. Because the KG1 cells were electroporated and put into media that was lacking G418 for the apoptosis experiments, there would be no need for the activation of the SV40 promoter allowing the CMV to transcribe the *hiwi* gene unperturbed, thus allowing for the rapid induction of apoptosis resulting in eventual cell death. This study indicates that *hiwi* inhibits KG1 cell proliferation and therefore suggests that this gene may play a role in the negative regulation of hematopoietic cells.

EXAMPLE 4

Cell and Tissue Expression of Human *hiwi* Gene

Human *hiwi* gene expression was assayed in a variety of human cells and tissues.

To determine the expression pattern of *hiwi* in various adult and fetal tissues other than $CD34^+$ marrow cells, PCR amplification was performed using *hiwi* specific primers on cDNA samples (Clontech Laboratories). Fetal cDNA samples ranged from 18 to 36 weeks of gestation. The expression level was determined through semiquantitative PCR amplification. PCR amplification was performed on 3 different Multiple Tissue cDNA Panels: human I, human II, and human fetal (Clontech Laboratories). The following PCR conditions were used for amplification with primer pair:

GSP2F4 (forward primer, 5'-CCTTGCCAGTACGCCCACAAGCTG-3' (SEQ ID NO. 16) and

GSP1R1966 (reverse primer, 5'-CCCCACCTATGGTTGTAGTGAGCATCC-3'

(SEQ ID NO. 17)

PCR amplification conditions used were: 1 cycle at 94°C/2 min; 35 cycles at 94°C/10s, 70°C/15s, 72°C/45s and 1 cycle at 72°C /10min which produced a 557 bp product. Positive samples were separated on a 1% SeaPlaque Agarose (FMC) 1x TAE gel stained with ethidium bromide and purified from agarose using the Wizard PCR Prep Kit according to the manufacturer's instructions. Asymmetric restriction endonuclease digestion was performed to determine the identity of the PCR products.

The results of this analysis revealed a wide distribution of *hiwi* through most fetal and adult tissues (Figures 7A and 7B). The highest level of expression in fetal tissues (Figure 7A) was found in the kidney. Analysis of adult samples (Figure 7B) showed that *hiwi* was also expressed in a wide range of tissues such as the prostate, ovary, small intestine, heart, brain, liver, skeletal muscle, kidney and pancreas. The highest level of expression was seen in the testis followed by the kidney. Expression of *hiwi* was not detected in mesenchymal stem cells or marrow stroma.

Human leukemia cell lines were also assayed for human *hiwi* expression. Because leukemia is frequently accompanied by expansion of the hematopoietic compartment, it was anticipated that leukemia cells would express *hiwi*, a gene that is associated with self-replication. A variety of immortalized human leukemia cell lines belonging to various lineages were examined; these results are shown in Figure 3A through 3C. Analysis of eight immortalized leukemia cell lines showed that the *hiwi* mRNA transcript was not detectable by RT-PCR. These results suggested that *hiwi* gene expression may not be a component of the genetic program that accompanies leukemogenesis. As disclosed above, *hiwi* gene expression appears to be limited within the hematopoietic compartment to normal $CD34^+$ cells. This data indicates that the lack of *hiwi* expression may be a consequence of and a marker for the leukemic transformation event.

The effect on $CD34^+$ expression of cell differentiation was also examined. $CD34^+$ cell differentiation was promoted in a suspension culture system to which SCF, IL-3, and G-CSF were added each at 100 ng/mL every 3-4 days. Bazil and colleagues (Bazil *et al.*, 1995, *Blood* 86: 502-511) have previously shown these conditions allow for rapid proliferation and differentiation causing the cells to leave the $CD34^+$ compartment. Aliquots of cells were harvested on days 0, 1, 3, 5, 7, 10, and 14 and analyzed flow cytometrically for $CD34^+$ expression and by semi-quantitative RT-PCR for the *hiwi* messenger RNA (mRNA) (results shown in Figure

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8A). On day 0, the starting cell population was composed of 99% CD34$^+$ cells. Day 5 CD34$^+$ content diminished to 20% of the expanded cell population; by day 7, fewer than 0.1% of the cells were CD34$^+$ while by day 10 there were no detectable CD34$^+$ cells present. By day 3, *hiwi* expression was markedly reduced and no longer detectable by semiquantitative RT-PCR by day 5. Concurrently β_2 microglobulin gene expression was used as an internal control. β_2 microglobulin levels remained constant throughout the 14 days of culture (Figure 8B).

These results indicate that *hiwi* expression is unique to the more primitive cellular compartment and that its expression might serve as a genetic marker of progenitor and stem cells.

EXAMPLE 5

Chromosome Mapping of the Human *hiwi* Gene

The chromosome location of *hiwi* was determined by Sequence Tagged Site (STS) computer based mapping (Taymans *et al.*, 1999, *Genomics* 56:344-349; Stringham *et al.*, 1999, *Am J Hum Genet.* 65:545-553). Four different STS clones (Genbank accession numbers AA639672, AA904973, AA969660, and AI25224) were found to have significant homology to the *hiwi* gene. These 4 clones, along with the partial published *hiwi* sequence were then mapped to the reference interval of D12S340-D12S97 (147.5- 160.9cM) by radiation hybridization (Unigene cross-reference Hs. 128673). The physical position of *hiwi* was located at 489.71 cR3000 (P1.43, stSG53541) on the q arm of chromosome 12, specifically between 12q24.2 through 12q24.32 as represented by the cytogenetic ideogram.

To directly determine the chromosomal location of the human *hiwi* genetic locus, a *hiwi* genomic clone was hybridized on metaphase chromosomes derived from a human peripheral blood cell culture. Identification of the human chromosomes was based on their DAPI-banding pattern that resembles G-bands achieved by conventional trypsin-Giemsa treatment. Thirty two metaphase spreads were analyzed; a representative spread is shown in Figure 9. These results showed specific localization to chromosome 12 as follows: specific hybridization signals were seen 9 times (28.1 %) in two chromatids, 10 times (31.3 %) in three chromatids and 11 times (34.4 %) in four chromatids, no hybridization signals were seen in two metaphases (6.2 %). A very small number of non-specific hybridization sites were seen (9).

Traditional FISH analysis confirmed the computer based chromosomal localization of *hiwi*.

The physical locale of *hiwi* does not currently show an association with any hematological disorders (as demonstrated by STS/EST homology comparisons).

EXAMPLE 6

Transduction of Human Hematopoietic Stem Cells

Human hematopoietic stem cells were transduced using a recombinant retrovirus to induce constitutive human *hiwi* gene expression introduced in the cells.

Retrovirus encoding human *hiwi* were produced by cloning the human *hiwi* gene described in Example 2 into a bicistronic mouse stem cell virus (MSCV) based vector genome that encodes the yellow fluorescent protein (YFP) under the control of the internal ribosome entry site (IRES). Producer cells were derived from the PG13 packaging cell line obtained from the American Type Culture Collection (ATCC; Manassas, VA). Producer cells were generated by transfecting the cells with *hiwi* and YFP-encoding cloned retrovirus. Retroviral vector particles were pseutotyped with the gibbon ape leukemia virus (GALV) envelope protein. The vector producing cell line was initially grown in Dulbecco's modified Eagle's medium (DMEM; BioWhittaker) containing 10% FBS and then slowly adapted to IMDM containing 10% FBS. Cells were cultured at 37^0C in 5% CO_2.

Human bone marrow cell aspirates were prepared as described in Example 1. $CD34^+$ purified cell populations were cultured in IMDM supplemented with 10% FBS, 2mM L-glutamine, 100U/mL penicillin, and 1mg/mL streptomycin. Cells were cultured for 24 hours at 37°C in 5% CO_2 before transduction in the presence of the following cytokines: stem cell factor (SCF; 300ng/mL), Flt-3 ligand (300ng/mL), interleukin-3 (IL-3; 10ng/mL) and interleukin-6 (IL-6; 10ng/mL); all cytokines were obtained from R&D Systems, Minneapolis, MN. Immunoselected cells were then plated at a density of 2-4 x 10^4 cells per well of a 48 well plate coated with 20ug/cm^2 recombinant human FN fragment CH-296 (Retronectin; Panvera, Madison, WI). Fresh retroviral supernatant and cytokines were added to the cells once a day for 4 consecutive days. On the last day of culture, cells were harvested and then analyzed flow cytometrically for CD34 and YFP expression.

Transduced human $CD34^+$ cells were analyzed for CD34 and YFP expression.

Cells were stained with anti-CD34 MoAb conjugated to allophycocyanin (APC; Becton Dickinson). Control cells were incubated with fluorochrome conjugated isotype matched IgG1-APC (Becton Dickinson). Prior to sorting, 1ug/mL propidium iodide (PI) was added to each sample to identify and exclude nonviable cells. Cells

5   were sorted on a FACSVantage cell sorter (Becton Dickinson); APC was excited at 633 nm and YFP expression was determined at an excited wavelength of 488 nm using an argon ion laser. Positive fluorescence for each of the markers was established as fluorescence greater than 99% of isotype-matched irrelevant murine IgG$_1$ controls. Cell aggregates or debris were excluded by forward and 90° light

10   scatter. All staining for analysis and sorting was done in the presence of 0.2% BSA in PBS on ice.

The results of flow cytometric analysis of a CD34$^+$ cell population that was transduced with the *hiwi*-containing retrovirus revealed an increase in the number of cells that retained the primitive CD34 antigen as well as the YFP expression marker.

15   This approximate 7% retention of primitive stem cells was derived from comparing the *hiwi* expressing population to an identically transduced population containing the MSCV retroviral empty vector. Figure 10 shows flow cytometric analysis demonstrating virtually no CD34$^+$YFP$^+$ cells in the empty vector control (minus the background from the empty vector isotype control) while the *hiwi*-containing vector

20   shows a seven fold increase (minus the background from the hiwi isotype control). This analysis was based upon approximately 7,000 gated events.

It should be understood that the foregoing disclosure emphasizes certain specific embodiments of the invention and that all modifications or alternatives equivalent thereto are within the spirit and scope of the invention as set forth in the

25   appended claims.